Cytokine and immunoglobulin subclass responses of rats to infection with Eimeria nieschulzi

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Abstract: SIV rats infected with a high dose (50000 oocysts) of Eimeria nieschulzi displayed clinical symptoms of coccidiosis such as diarrhoea (days 6 and 7 post-primary infection) and weight loss (days 6-8 post-primary infection) and were completely immune to challenge with a similar dose. The ability of rats to produce tumour necrosis factor (TNF) in vivo was enhanced during the period of oocyst excretion in the primary infection but significant production of TNF did not occur after challenge infection. Thus, TNF does not appear to be an important factor in resistance to infection with E. nieschulzi but may play some role in resistance to primary infection and in the pathology associated with E. nieschulzi infection. Parasite-specific serum IgM levels (measured by enzyme-linked immunosorbent assay) were also increased during primary infection but returned to background levels at the end of the patent period and were not affected by challenge infection. In contrast to TNF and IgM, serum concentrations of E. nieschulzi-specific IgGl, IgG2a, IgG2b, IgG2c and intestinal tissue levels of IgA did not begin to increase until after day 12 post-primary infection, reached peak levels between days 20 and 30 post-primary infection and were slightly increased by challenge infection

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Cytokine and immunoglobulin subclass responses of rats to infection with *Eimeria nieschulzi*

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SUMMARY

SIV rats infected with a high dose (50000 oocysts) of *Eimeria nieschulzi* displayed clinical symptoms of coccidiosis such as diarrhoea (days 6 and 7 post-primary infection) and weight loss (days 6–8 post-primary infection) and were completely immune to challenge with a similar dose. The ability of rats to produce tumour necrosis factor (TNF) *in vivo* was enhanced during the period of oocyst excretion in the primary infection but significant production of TNF did not occur after challenge infection. Thus, TNF does not appear to be an important factor in resistance to infection with *E. nieschulzi* but may play some role in resistance to primary infection and in the pathology associated with *E. nieschulzi* infection. Parasite-specific serum IgM levels (measured by enzyme-linked immunosorbent assay) were also increased during primary infection but returned to background levels at the end of the patent period and were not affected by challenge infection. In contrast to TNF and IgM, serum concentrations of *E. nieschulzi*-specific IgG1, IgG2a, IgG2b, IgG2c and intestinal tissue levels of IgA did not begin to increase until after day 12 post-primary infection, reached peak levels between days 20 and 30 post-primary infection and were slightly increased by challenge infection.

Key words: *Eimeria nieschulzi*, tumour necrosis factor (TNF), immunity, pathology, antibodies.

INTRODUCTION

Direct evidence that TNF is produced in response to *Eimeria* has recently been presented (Byrnes *et al.* 1993). Thus, splenic macrophages from chickens infected with either *Eimeria tenella* or *Eimeria maxima* produce TNF upon *in vitro* stimulation with lipopolysaccharide (LPS) in a biphasic manner corresponding, firstly, to the time when pathology associated with infection is most obvious and, secondly, to the time when effective immunity to challenge infection normally develops. Additionally, early TNF production is much greater in macrophages from chickens infected with the pathogenic but relatively less immunogenic species, *E. tenella*, compared to the relatively less pathogenic but extremely immunogenic, *E. maxima*, leading the authors (Byrnes *et al.* 1993) to speculate that high levels of TNF are associated with the pathology associated with coccidiosis whereas more moderate TNF production is associated with the development of resistance. Indeed, many of the pathological manifestations of coccidiosis, including glycogenolysis, depletion of lipid stores, dehydration, acidosis, diarrhoea, muscle breakdown, intravascular coagulation, increased concentrations of stress hormones, fever, extravasation of polymorphonuclear leukocytes and intestinal vascular leakage, are hallmark effects of TNF production in other experimental systems (reviewed by Byrnes *et al.* 1993). However, in the study of Byrnes *et al.* (1993), no effort was made to correlate directly any of these pathological parameters with production of TNF *in vivo* nor were the effects of challenge infection with *Eimeria* species on TNF production reported. Thus, in this study, we have attempted to correlate *in vivo* production of TNF with the grosser effects of infection with the rat coccidian, *Eimeria nieschulzi*, such as weight loss and diarrhoea. We have also attempted to measure *in vivo* production of gamma-interferon (IFN) in response to this parasite, since this cytokine is considered to play a central role in resistance to *Eimeria* spp. (Rose & Wakelin, 1989; Wakelin & Rose, 1990; Lillehoj & Trout, 1994) and looked for an association between cytokine production and the production of specific immunoglobulin subclasses in response to both primary and secondary infections.

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MATERIALS AND METHODS

Animals and parasites

Female SIV rats, aged 7–8 weeks, which were raised at the University of Zurich, were used exclusively in

this study. Throughout the study the rats had free access to food and water and were housed at 21 °C with a 12 h light/dark cycle. *E. nieschulzi* oocysts were originally obtained from Dr R. Entzeroth (University of Bonn). Rats to be infected were lightly anaesthetized with methoxyflurane and then inoculated by stomach tube with different doses of *E. nieschulzi*. Rats were weighed throughout the study and the occurrence of diarrhoea noted. The rats weighed approximately 240 g at the time of infection.

Cytokine assays

IFN and TNF levels in the plasma of infected and uninfected rats were measured as previously described and validated (Smith & Ovington, 1994). IFN and TNF concentrations in intestinal supernatants were also determined. The intestinal samples were prepared as follows: after exsanguination, the small intestines were removed, flushed with ice-cold phosphate-buffered saline (pH 7·2) containing 1 mM phenylmethylsulfonylfluoride (Sigma) and then homogenized with an electrical homogenizer in a final volume of 15 ml using the same solution. The intestinal homogenates were then centrifuged at 12000 ***g*** for 15 min at 4 °C and the supernatant fractions stored at −20 °C until assayed for cytokines.

Antibody responses to Eimeria nieschulzi

Enzyme-linked immunosorbent assays (ELISAs) using soluble sporozoite antigen extracts were performed on serum and intestinal supernatant samples as previously described (Smith *et al.* 1993). Antibody responses were measured by ELISA using soluble antigen from purified sporozoites since the results of experiments in which the parasite life-cycle was abbreviated by treatment with Baycox at 80 h post-primary infection demonstrated that immunity was induced by the asexual stages (data not shown). Serum samples were diluted 1:100 for all isotypes except IgM and IgG2b which were diluted 1:1000 and intestinal supernatants were used at 1:10 dilutions. Alkaline phosphatase-conjugated antibody to the various rat immunoglobulins (The Binding Site, UK) were used at 1:500 dilutions for IgM, IgG2a and IgG2b and at 1:50 for IgA, IgG1 and IgG2c.

Oocyst counts

Faeces were collected from individually caged rats daily from days 5–15 post-infection. The daily collection of faeces from each rat was homogenized in saturated saline to a final volume of 50 ml using a Waring blender. Samples of these homogenates were diluted a further 1:10 with saturated saline when necessary. Duplicate subsamples were counted under a microscope using a modified McMaster chamber slide.

Experimental design

Based on the results of a pilot experiment (data not shown) showing that only a very high primary inoculum induced any pathological effects as well as complete abrogation of oocyst excretion upon challenge, 117 rats were infected with 5×10^4 sporulated oocysts of *E. nieschulzi*. Of these, 5 rats were individually caged and faeces collected daily to determine oocyst excretion. The remaining rats were caged in pairs. Eight infected rats and 8 uninfected rats were used to monitor body weight change and the incidence of diarrhoea. The remaining 96 rats were used to determine intestinal and serum levels of TNF, IFN and parasite-specific immunoglobulins at different times post-infection and post-challenge. Eight rats were killed at each time-point for collection of sera and intestinal samples; 4 of these 8 rats were injected intraperitoneally with 1 mg of LPS (Sigma; phenol extracted from *Escherichia coli* serotype O128:B12) 90 min prior to necropsy. LPS was used to enhance the TNF response since TNF is often difficult to detect in tissue samples and serum due to its short half-life and rapid binding to abundant receptors.

Statistical analysis

Data were analysed by analysis of variance (ANOVA) using one-way or two-way with interaction, as appropriate. Differences at the $P < 0{\cdot}05$ level were taken to be significant. Student's *t*-tests were applied, when required, using the standard error of the differences of the means calculated from the ANOVA. Again, differences at the $P < 0{\cdot}05$ level were taken to be significant.

RESULTS

Oocyst excretion following primary infection with *E. nieschulzi* began on day 7 and peaked on day 9 post-primary infection before declining rapidly each day thereafter so that no oocysts could be detected in any of the faecal samples by day 13 post-infection (Fig. 1A). There was no detectable oocyst excretion following secondary infection.

Infected rats began to lose weight almost immediately following primary infection though greatest weight loss occurred between days 6 and 8 post-primary infection (Fig. 1B) with the result that the body weight of infected rats was significantly lower than that of control rats from day 6 post-primary infection until the end of the experiment on day

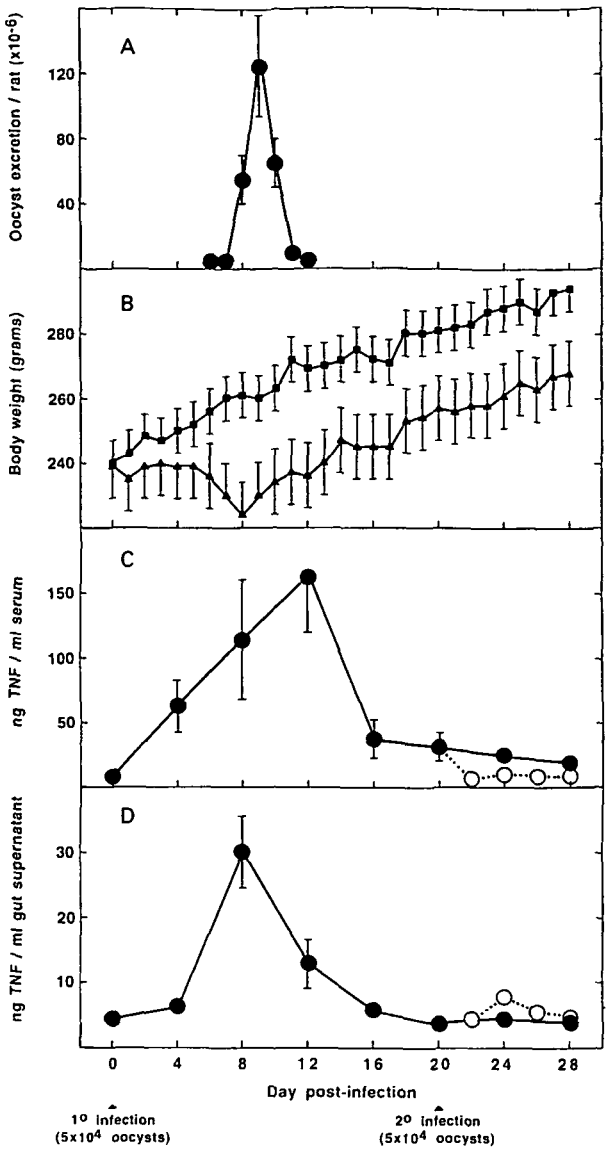

Fig. 1. Oocyst excretion (A), body weight (B) and TNF concentrations in sera (C) and supernatants from intestinal homogenates (D) of rats infected with *Eimeria nieschulzi*. Results are means ± S.E.M. For oocyst excretion, $n = 5$ rats. For body weight, $n = 8$ for infected rats (▲) and 8 for uninfected rats (■). For TNF concentrations, $n = 4$ for rats injected intraperitoneally with LPS, 90 min prior to necropsy. (●) Rats exposed to a single infection only; (○) rats exposed to 2 infections.

28 ($P < 0{\cdot}05$, two-way ANOVA with interaction, Student's *t*-test). Seventy-five percent (6 of 8) rats suffered diarrhoea on days 6 and 7 post-primary infection but no rats had diarrhoea at any time after challenge infection.

IFN could not be detected in intestinal or serum samples of any rats, whether treated or not with LPS prior to necropsy. TNF was also not detectable in intestinal or serum samples of rats not treated with LPS. However, TNF was detected in the intestines and sera of all rats injected with LPS prior to necropsy and the levels of TNF detected varied depending on the time after infection with *E. nieschulzi* that the samples were taken (Fig. 1C and D). Serum concentrations of TNF in rats treated with LPS increased in a linear fashion soon after primary infection with peak levels being observed on day 12 post-primary infection (Fig. 1C; TNF levels significantly greater than day 0 values on days 4, 8 and 12 post-primary infection, $P < 0{\cdot}05$, one-way ANOVA, Student's *t*-test). Subsequently, serum TNF levels in infected rats were not significantly different from uninfected (day 0) rats and challenge infection at 20 days post-primary infection did not increase the ability of the rats to produce TNF in response to LPS. In fact, TNF levels in the sera of challenged rats were slightly lower than those seen in the sera of rats not challenged with *E. nieschulzi* (Fig. 1C). Intestinal concentrations of TNF in rats injected with LPS followed a similar pattern to that seen in sera except that peak levels were observed on day 8 post-primary infection rather than day 12 (Fig. 1D).

The injection of LPS prior to necropsy had no effect on serum or intestinal antibody levels in rats infected with *E. nieschulzi*. Therefore, these 2 groups were combined into single data sets comprising 8 rats per time-point (Fig. 2). Significant levels of IgA could not be detected in any serum samples from rats infected with *E. nieschulzi*. However, supernatants from intestinal homogenates contained parasite-specific IgA (Fig. 2), with increased levels being detected sequentially from day 12 to day 28 post-primary infection (day 28 = day 24 > day 20 = day 16 > day 12 = day 8 = day 4 = day 0, $P < 0{\cdot}05$, one-way ANOVA, Student's *t*-test). Further slight increases in anti-*E. nieschulzi* IgA levels were induced by challenge infection, though these were not statistically significant.

Parasite-specific serum IgM levels peaked relatively early after primary infection (by day 8) then declined to levels only slightly greater than control values. Challenge infection had little effect on anti-*E. nieschulzi* IgM levels (Fig. 2). An identical pattern of IgM concentrations was observed in the supernatants of the intestinal homogenates and, therefore, these data are not reproduced here.

In contrast to IgM, parasite-specific IgG subclass (IgG1, IgG2a, IgG2b, IgG2c) levels did not begin to increase ($P < 0{\cdot}05$, one-way ANOVA, Student's *t*-test) until after day 12 post-primary infection and peaked at a relatively late time after primary infection (day 24, 28, 24 and 20, respectively). Challenge infection at day 20 post-primary infection induced slightly (but not statistically significantly) increased production of *E. nieschulzi*-specific IgG1, IgG2a and IgG2b but not IgG2c, which was not increased by challenge infection (Fig. 2). As was the case with IgM, intestinal homogenate supernatant levels of anti-parasite IgG antibodies almost exactly paralleled the response pattern seen in sera.

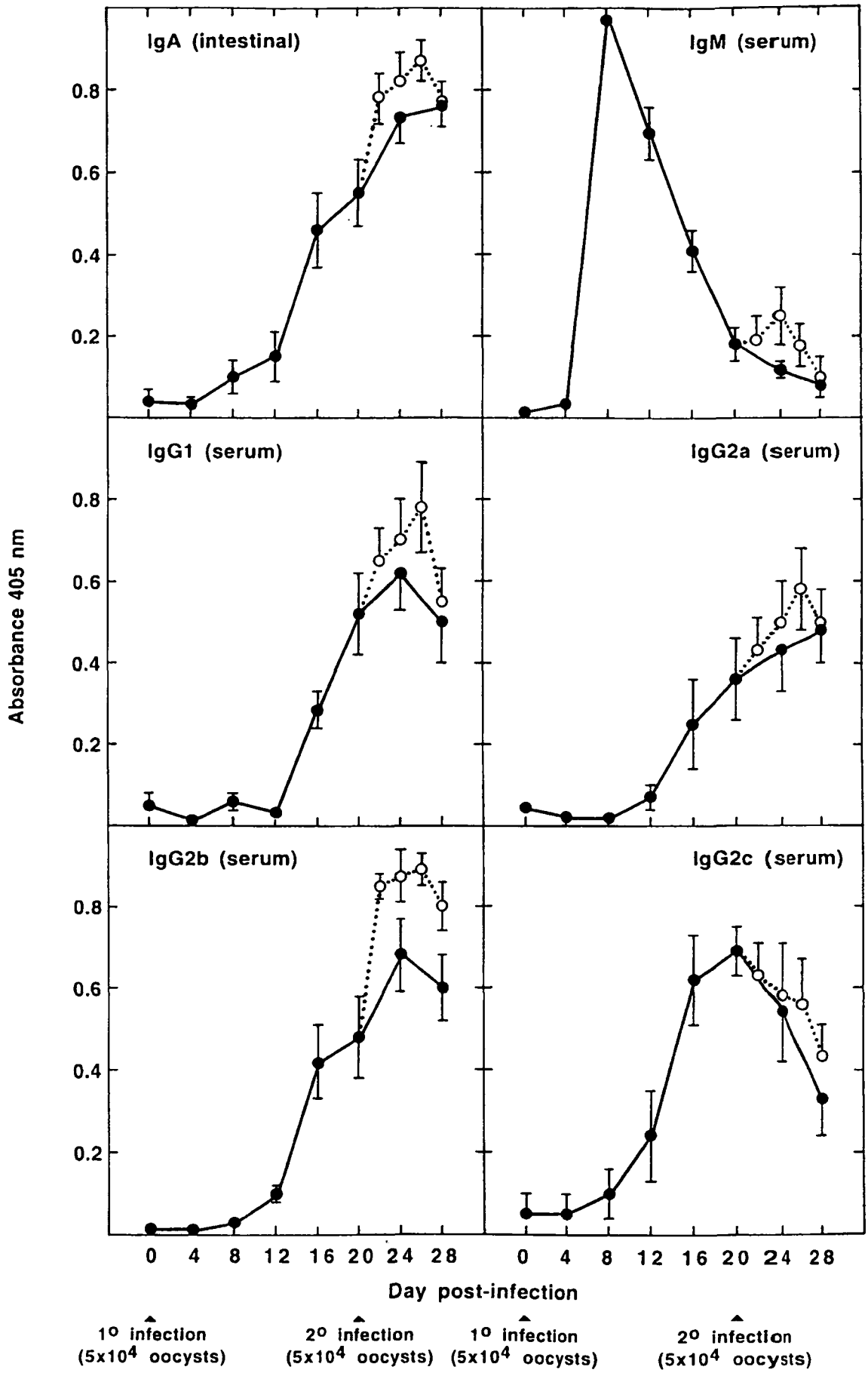

Fig. 2. For legend see opposite.

DISCUSSION

Byrnes *et al.* (1993) have proposed that resistance to reinfection with *Eimeria* species may be related to the production of TNF. Immunity to, for example, *E. nieschulzi* is certainly T cell dependent as demonstrated by the inability of congenitally athymic (nude) rats to resist infection (Rose *et al.* 1979). However, the ability of rats infected with *E. nieschulzi* to produce TNF *in vivo* (for example, in response to LPS) is actually slightly reduced by challenge infection, indicating that other factors are more important in the expression of immunity. It is conceivable that TNF may be involved in resistance to primary infection with *E. nieschulzi* since the ability of rats to produce TNF, particularly in the small intestine, is greatest during the patent period of infection (Fig. 1). Although the course of primary infection with *E. nieschulzi* (and numerous other coccidia) is little affected by the immune system (Rose, 1987) indicating that the life-cycle of *E. nieschulzi* is determined mainly by genetic constraints of the parasite, significantly more oocysts are excreted by nude rats compared to euthymic rats indicating some degree of immune control of primary infections (Rose *et al.* 1979).

The pathology associated with infection with *E. nieschulzi* is also at least partially T cell dependent. Thus, the crypt hyperplasia and villous atrophy, cellular infiltration and oedema which occur in the intestines of immunologically intact rats infected with *E. nieschulzi* is not nearly as severe in athymic rats (Rose & Hesketh, 1982). Similar results have been obtained in helminth-infected animals (Ferguson & Jarrett, 1975) and, furthermore, nude rats infected with the nematode, *Nippostrongylus brasiliensis*, do not experience the same degree of cachexia, anaemia or diarrhoea as euthymic rats (Smith, Ovington & Bryant, 1991). The appearance of TNF in the serum of rats infected with *N. brasiliensis* correlates with the period when pathology is most evident (Smith & Ovington, 1994) indicating a potential role for this cytokine in the immunopathology of enteric infections, as previously forecast by Ovington (1987) based on the changes in protein, carbohydrate and lipid metabolism apparent in infected rats. Similarly, Byrnes *et al.* (1993) have proposed that high levels of TNF produced in response to coccidia may contribute to pathology. This proposal would seem to have some merit in the light of our findings that the ability of rats heavily infected with *E. nieschulzi* to produce TNF, upon *in vivo* stimulation with LPS, is greatest at about the time that weight loss is most apparent and when most rats have diarrhoea. However, some caution must be exercised in this interpretation since TNF could not be detected in the sera or intestinal preparations of rats heavily infected with *E. nieschulzi* but not treated with LPS, in contrast to rats heavily infected with *N. brasiliensis* where average serum concentrations of TNF of over 300 pg/ml were detected immediately before the period of worst pathology (Smith & Ovington, 1994). Similarly, TNF is detectable in the sera of mice (Grau *et al.* 1987; Clark & Chaudri, 1988) and humans (Scuderi *et al.* 1986; Grau *et al.* 1989; Kwiatkowski *et al.* 1990) in concentrations of thousands or hundreds of pg/ml in association with immunopathology due to *Plasmodium* infections.

IFN is generally regarded (Rose & Wakelin, 1989; Wakelin & Rose, 1990; Lillehoj & Trout, 1994) as a major player in resistance to *Eimeria* because it inhibits cell invasion and development of the parasite *in vitro*, is produced by lymphocytes isolated from infected animals, and administration of monoclonal antibody to IFN effectively renders mice that are normally quite resistant to primary infection, fully susceptible. Our inability to detect IFN in sera or intestines of rats infected with *E. nieschulzi* cannot in the light of previous work with other *Eimeria* species, be regarded as evidence for lack of involvement of IFN in resistance to *Eimeria*. However, IFN is detectable in sera of mice (Slade & Langhorne, 1989) and humans (Kwiatkowski *et al.* 1990) infected with *Plasmodium* and serum IFN levels are significantly correlated with serum TNF concentrations (Kwiatkowski *et al.* 1990). It is therefore perhaps slightly surprising that IFN was not detected if it does, in fact, play an important role in resistance to *E. nieschulzi*.

As previously reported (Rose, Peppard & Hobbs, 1984), infection with *E. nieschulzi* induces the appearance of specific antibodies of the IgM and various IgG subclasses in the blood circulation. The kinetics of the circulating immunoglobulin responses in our study were similar to those observed by Rose *et al.* (1984) in that serum levels of *E. nieschulzi*-specific IgM peaked at a relatively early time post-primary infection, whereas all the IgG subclasses (including IgG2c, which was not measured by Rose *et al.* 1984) reached peak serum concentrations between days 20 and 30 post-primary infection. Rose *et al.* (1984) reported an anamnestic response of IgG antibodies to challenge infection, though they did not actually compare the effects of challenge to a similarly prolonged primary infection. In our study, there was little evidence of an anamnestic IgG response, with only slight increases in the antibody

Fig. 2. Serum (IgM, IgG1, IgG2a, IgG2b, IgG2c) and intestinal (IgA) antibody responses of rats to infection with *Eimeria nieschulzi*. Results are means ± S.E.M., $n = 8$ rats. (●) Rats exposed to a single infection only; (○) rats exposed to 2 infections.

concentrations following challenge. However, the timing of challenge (day 20 post-primary infection) was around the time of peak IgG responses to primary infection so that any boosting effect of challenge infection may have been obscured.

Parasite-specific IgA antibodies were detected in intestinal samples but not in sera of rats infected with *E. nieschulzi*. However, the kinetics of IgA appearance were strikingly different from those reported by Rose *et al.* (1984) who found peak levels of IgA in bile on days 7 and 8 post-primary infection. In contrast, we have found that intestinal tissue levels of *E. nieschulzi*-specific IgA were not significantly elevated above control values until as late as 12 or 16 days post-infection with peak levels being reached between 24 and 28 days post-primary infection. As with the IgG subclasses, IgA levels were little affected by challenge infection but, again, were already present in high concentrations at this time. The reasons for this discrepancy between the 2 studies are not known.

The production of specific immunoglobulin subclasses does not correlate with either IFN or TNF production in rats infected with *E. nieschulzi*, since IFN was not detected and TNF production was enhanced relatively early after primary infection but antibodies (except IgM) were produced much later. A particularly intriguing observation with regard to the antibody response to *E. nieschulzi* was that IgG1, IgG2a, IgG2b and IgG2c antibodies shared very similar kinetics. In mice, the association of IgG1 and IgE antibodies with T helper 2 (TH2) cells (which cause production of interleukins 3, 4, 5, 6 and 10) and the association of IgG2a antibodies with TH1 cells (which cause production of IFN, TNF, lymphotoxin, granulocyte/macrophage colony stimulating factor and interleukins 2 and 3) is well documented (Mosmann & Coffman, 1989). More precisely, interleukin 4 apparently controls production of IgG1 and IgE antibodies and IFN controls production of IgG2a antibodies mutually exclusively. Evidence also exists to suggest that interleukin 5 influences IgA production (Else & Grencis, 1991) though recent data indicate that transforming growth factor beta is an important factor in the regulation of IgA production (Snapper & Mond, 1993). Although not as intensively studied, a similar association of cytokines and antibody subclass production is apparent in rats (Uchikawa *et al.* 1994). Thus, IgE and IgG1 antibodies are produced in close association by rats in response to *N. brasiliensis* and the production of these Ig subclasses is dissociated from production of IgG2a (Yamada, Nakazawa & Arizono, 1993). Furthermore, increased IgG2a production is associated with IFN whereas increased IgE production is associated with suppression of IFN production in parasitized rats (Uchikawa *et al.* 1994). No evidence for a dichotomous TH cell response can be found in the antibody response to *E. nieschulzi* since the kinetics of appearance of all IgG subclasses and IgA is virtually identical and evidence for the production of IgE antibodies in response to *E. nieschulzi* is equivocal (Rose *et al.* 1984). That a dichotomous TH cell response to *E. nieschulzi* is not readily apparent is perhaps not surprising since even TH cells from mice infected with *Eimeria vermiformis* produce IFN and interleukins 5 and 10 with very similar kinetics during infection (Wakelin *et al.* 1993). Furthermore, resistant mice produce much greater quantities of the TH2 cytokines, interleukins 5 and 10, than do susceptible mice (Wakelin *et al.* 1993) despite the fact that resistance is apparently mediated by the TH1 cytokine, IFN (Rose, Wakelin & Hesketh, 1989, 1991), of which interleukin 10 is a known antagonist (Mosmann & Moore, 1991). All of this indicates that the TH1/TH2 dichotomy so characteristic of murine leishmaniasis (Reed & Scott, 1993) may not function in the same way for *Eimeria* infections.

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